#35

Exhibit A

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Burrell	Confirmation No.:	4511
Serial No.:	08/284,199	Art Unit:	1638
Filed:	August 2, 1994	Examiner:	David Fox
For:	MODIFICATION OF PLANT METABOLISM	Attorney Docket No:	9341-005

DECLARATION OF STEPHEN ANDREW COATES UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, STEPHEN ANDREW COATES, do declare and state that:

1. I am a citizen of the Great Britain, residing at 1 Harcombe Road, Cherry Hinton, Cambridge, CB1 9PD, United Kingdom.

2. I am a colleague of inventor Dr. Michael M. Burrell of the invention described and claimed in the above-identified patent application, Application No. 08/284,199 ("the '199 application").

3. I am currently a project leader at Advanced Technologies (Cambridge) Ltd. (hereafter "ATC"). ATC is the Assignee of the above-identified application. From 1997-2000, I was a senior research scientist at ATC and from 1990-1997, I was a research scientist at ATC.

4. My academic and technical experience and honors, and a list of my publications are set forth in my curriculum vitae, which is attached hereto as Appendix 1.

5. I have read and am familiar with the '199 application. The '199 application teaches transforming plants with a chimeric gene comprising a nucleotide sequence encoding pyruvate kinase, acid invertase, starch synthase, 6-phosphofructokinase, sucrose synthase, and/or sucrose phosphate synthetase operably linked to a promoter that directs the desired expression. The transformed plants exhibit a modification in the amount

of a metabolic intermediate in the glycolysis, starch synthesis, starch degradation, sugar synthesis, or sugar degradation pathway. The invention also encompasses methods for making such transgenic plants, the transgenic plants, chimeric genes, and transgenic tubers and seeds.

6. I have read and am familiar with the pending claims and the outstanding Office Action dated January 13, 2003 for the '199 application. I have been informed and believe that the claims of the '199 application are subject to a rejection based on the contention that the '199 application allegedly does not provide sufficient guidance for making and using a transgenic plant with the above mentioned chimeric genes comprising pyruvate kinase, acid invertase, starch synthase, 6-phosphofructokinase, sucrose synthase, and/or sucrose phosphate synthetase in any plant other than potato, and that methods for making such plants and the plants themselves would require undue experimentation.

7. The following experiment was conducted by myself, Emma Louise Schofield, a student under the supervision of my colleague Dr. Michael Burrell at ATC, and Robin Dunford, a student, also under the supervision of Dr. Michael Burrell. The first experiment described below shows that plants transformed with a chimeric gene comprised of a nucleic acid molecule that is the antisense to the sequence coding for invertase enzyme operably linked to the patatin promoter exhibit both a modification in invertase activity and a modification in the content of sugars in comparison with non-transformed control plants. In the second experiment, plants transformed with an antisense sucrose synthase sequence under the control of a patatin promoter and plants transformed with an sense sucrose synthase sequence under the control of a patatin promoter both exhibited a modification in sucrose synthase activity and a modification in the amount of metabolic intermediates (sucrose, glucose, and fructose) in comparison non-transformed control plants. The third experiment demonstrates that plants transformed with a nucleic acid molecule encoding pyruvate kinase or the antisense thereof operably linked to a tuber specific promoter exhibit significantly reduced pyruvate kinase activity in comparison to non-transformed control plants. The fourth experiment demonstrates that plants transformed with nucleic acid molecules encoding both pyruvate kinase (PK) and phosphofructokinase (PFK) exhibit decreases in the activity of both enzymes. Taken together, these results are supportive of and predict that one skilled in the art of plant transformation could follow the teachings of the specification and make and use the methods and plants of the invention without engaging in undue experimentation.

8. The first experiment was conducted to determine if plants could be

successfully transformed with a chimeric gene encoding invertase oriented in the antisense direction, which would modify invertase expression, and whether or not such plants with modified invertase expression would exhibit modified invertase activity and sugar content. Potato plants were transformed with an antisense acid invertase gene sequence under the control of the tuber specific patatin promoter using the binary vector pBin19 which harbored the constructs, see Figure 1. Transformation was carried out using the technique as described in Appendix 3 for potatoes. A population of 80 transformed potato plants were generated. Of these, 8 were selected on the basis of a high sucrose/reducing sugar ratio for a field trial. The three lines shown in Table 1 were included in this initial selection. The other lines were rejected after the first field trial on the basis of poor agronomic performance. The three lines selected exhibited significantly reduced acid invertase activity in microtubers. These lines were grown under field conditions for two successive years. Tubers were harvested and stored under different temperature regimes, including 40°C for 6 weeks, 40°C for 12 weeks, 60°C for 12 weeks, and 100°C for 12 weeks. Tubers were extracted after time in storage, and sugar contents were determined using the enzyme linked spectrophotometric assay method (further details of the experimental procedures and methods provided in Appendix 3). The results presented in Table 2 show that transformed plants had significantly decreased tuber glucose and fructose contents, measured as mean micromoles per gram fresh weight, compared to control plants over a two year period under varying storage temperatures and lengths. The sucrose contents of the tubers were not significantly different from control tubers. Yield was also recorded for the tuber yield at harvest of the antisense invertase lines in two field trials carried out in 1997 and 1998. The '97 trial is a mean based on up to 10 plots for each line consisting of 10 plants per plot, see Figure 2. The '98 trial is based on up to 5 plots for each line of 100 plants per plot, see Figure 3. The difference in number of plots accounts for the difference in absolute values between the yield results from '97 and '98. Statistically there were no significant reductions in yield compared to the control line. The absence of a significant difference in yield between transgenic and non-transgenic control plants is indicative of the lack of deleterious phenotypic effects in the transgenic plants.

9. For the second experiment, potato plants were transformed with a construct comprising either an antisense or a sense potato sucrose synthase sequence (endogeneous potato *Sus4* isoform) under the control of a tuber specific promoter (patatin PS3/27 promoter) using Bin19 based plasma vectors which harbored the constructs, Figures 4 and 5, and standard transformation methods (further details of the experimental procedures and methods provided in Appendix 3). Sucrose synthase activity was measured in

microtubers of transformed and control lines, as shown in Figure 6 (antisense) and Figure 7 (sense), some lines were found to have significantly lower enzyme activities than control lines. The three antisense lines and two sense co-suppression lines with the low sucrose synthase activities were isolated, see Figure 8. Plants having the sense construct and plants having the antisense construct were grown in both the greenhouse and in the field. Greenhouse grown tubers were pooled and it was determined that the glucose and fructose contents of the transformed lines were significantly higher than in control plants, see Figure 9. As shown in Figure 10, the glucose-6-phosphate and fructose-6-phosphate contents in the pooled tubers from transgenic plants were similar to control tubers, however the content of glucose-1-phosphate was significantly reduced in the tubers of transformed plants grown under greenhouse conditions.

10. These changes in metabolite levels were reflected in field grown plants where the three antisense lines with the lowest sucrose synthase activities selected in the greenhouse also exhibited reduced sucrose synthase activity when grown in the field as shown in Figure 11. The tuber glucose and fructose contents of the transformed lines were significantly higher than in control plants as shown in Figure 12. In comparison to control plants, transgenic tubers contained less starch and accumulated both sucrose and hexose, however these results were not associated with the development of a characteristic phenotype in the transgenic plants. Thus, the results of the second experiment demonstrate that transgenic plants with modified amounts of intermediates can successfully be made using the teachings of the specification.

11. The third experiment was designed to examine modification of the amount of intermediates in the starch and sugar pathways following transformation with a chimeric gene comprising a pyruvate kinase (PK) sequence (potato cytosolic PK) either in the sense or antisense direction under the control of a tuber specific promoter (patatin PS3/27 promoter). Potato plants were transformed with a construct comprising a pyruvate kinase sequence either in the sense or antisense direction under the control of a tuber specific promoter using the binary vector pBin19 which harbored the constructs, Figures 13d (antisense) and 13e (sense), and standard microtuber transformation methods (further details of the experimental procedures and methods provided in Appendix 3). Pyruvate kinase activity was determined as the NADH-oxidation in the presence of PEP and lactate dehydrogenase that was also dependent on the presence of ADP. The results presented in Figure 14 show that enzyme activities measured in pooled antisense and sense lines had

significantly decreased enzyme activities in comparison to non-transformed control plants. Three antisense and two sense transgenic lines having significant decreases in pyruvate kinase activity were further analyzed as a percent of the PK activity of control plants as shown in Table 3.

12. For the fourth experiment, the maximum catalytic activities of pyruvate kinase (PK) and phosphofructokinase (PFK) were measured in extracts of microtubers produced from shoots which were regenerated from leaf discs co-cultivated with the binary vector pFW4113 (encoding potato cytosolic PK, in sense direction) and pFW4023 (encoding *E. coli* PFK(ATP)). PK activity was determined as the NADH-oxidation in the presence of PEP and lactate dehydrogenase that was also dependent on the presence of ADP. PFK activity was determined as described by Kruger et al., 1989, Archives of Biochemistry and Biophysics 267:690-700. Figure 15 shows the mean enzyme activities for 26 transgenic lines, arranged in order of increasing activity. Error bars represent the standard deviation of the means, and the dashed lines represent the 95% confidence limits of the means of the control enzyme activities. The majority of the individual lines transfected with pFW4113 (encoding potato cytosolic PK, in sense direction) and pFW4023 (encoding *E. coli* PFK(ATP)) exhibited a decrease in enzyme activities that was within the means of the control activity. One transformed line exhibited a significant increase in PFK activity comparison to the control mean.

13. In view of the foregoing, I conclude, and others skilled in the art would also conclude, that a chimeric gene comprising a nucleotide sequence encoding pyruvate kinase, acid invertase, starch synthase, 6-phosphofructokinase, sucrose synthase, and/or sucrose phosphate synthetase can be transformed into and expressed in a plant to modify the amount of a metabolic intermediate in the glycolysis, starch synthesis, starch degradation, sugar synthesis, or sugar degradation pathway. The foregoing results demonstrate that one skilled in the art of plant transformation, following the teachings of the specification, can readily and successfully make and use the transgenic plants of the invention.

14. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: _____

_____
Stephen Andrew Coates

Attachments:

Appendix 1: *Curriculum Vitae* of Stephen Andrew Coates

Appendix 2: Tables 1 to 3 and Figures 1-15B.

TABLE 1. Invertase Activity In Mature Potato Tubers.
TABLE 2. Modification of Sugar Content in Transgenic Plants
TABLE 3. Modified Pyruvate Kinase Activity in Transgenic Plants
FIGURE 1 pFW14272
FIGURE 2 Tuber Yield 1997 For Field Trial of Antisense Invertase Lines
FIGURE 3 Tuber Yield 1998 For Field Trial of Antisense Invertase Lines
FIGURE 4 Patatin Antisense Tuber Sucrose Synthase Plasmid
FIGURE 5 Patatin Sense Tuber Sucrose Synthase Plasmid
FIGURE 6 Modified Sucrose Synthase Activity in Transgenic Plants
FIGURE 7 Patatin - Sense Tuber SS1 Construct Tuber Sucrose Synthase Activity
FIGURE 8 Tuber Sucrose Synthase Activity Screen
FIGURE 9A Tuber Glucose Content
FIGURE 9B Tuber Fructose Content
FIGURE 10A Potato Tuber Glucose-6-Phosphate
FIGURE 10B Potato Tuber Fructose-6-Phosphate
FIGURE 10C Potato Tuber Glucose-1-Phosphate
FIGURE 11 Tuber Sucrose Synthase Activity Screen
FIGURE 12A Tuber Glucose Content
FIGURE 12B Tuber Fructose Content
FIGURE 12C Tuber Sucrose Content
FIGURE 13A The Binary Vector pBin19, its GUS-Encoding Derivative pFW4101, and three PK-34-Containing Vectors Derived from pFW4101
FIGURE 13B pFW4101
FIGURE 13C pFW4111
FIGURE 13D pFW4112
FIGURE 13E pFW4113
FIGURE 14 PK Activity of Plants Transgenic for PK.
FIGURE 15A PK Activity of Transgenic Lines (PK+PFK)
FIGURE 15B PFK Activity of Transgenic Lines (PK+PFK)

Appendix 3 Materials and methods for experiments

CURRICULUM VITAE

STEPHEN ANDREW COATES

Personal Details.

Date of Birth. 14th September 1964.
Nationality. British.

Education.

1984-1987 Peterhouse, University of Cambridge.

- Entrance Exhibitioner reading for the B.A.(Hons.) degree in Natural Sciences.
- Awarded a College Scholarship, 1985.
- Awarded the 'John Worthington Scholarship in Natural Sciences', 1986.
- Awarded an upper second class B.A.(Hons.) (converted to M.A., 1991) degree in Natural Sciences (Part II Botany), 1987.

1987-1990 Botany School (*now* Department of Plant Sciences), University of Cambridge.

- Received a S.E.R.C. Quota award to study for the degree of Ph.D., under the supervision of Dr. T. ap Rees.

Thesis title : The Metabolism of Hexose Phosphates by Soybean Leucoplasts.

Thesis submitted December 1990, Ph.D. degree awarded June 1991.

Employment.

1990-1997	Research Scientist, Advanced Technologies (Cambridge) Ltd.
1997- 2000	Senior Research Scientist, Advanced Technologies (Cambridge) Ltd.
2000-present	Project Leader, Advanced Technologies (Cambridge) Ltd.

received
8/18/03

TABLE 1. Invertase Activity In Mature Potato Tubers.

Line	Activity (nmoles/min/g fresh wt)
Control	27.14 +/- 8.82
14272 24	3.4
14272 34	0.4
14272 69	0

TABLE 2. Modification of Sugar Content in Transgenic Plants

Year 1

Storage at 40°C for 6 weeks.

			umol/gram fresh wt.		
	construct	line no	GLUCOSE	FRUCTOSE	SUCROSE
Record	control	19	8.0736212	6.1126828	7.5382373
Record	14272	24	2.8166354	1.6225036	12.014392
Record	14272	34	4.5585355	3.2421482	10.581299
Record	14272	69	3.7374935	2.6442801	10.66786

Storage at 40°C for 12 weeks.

			umol/gram fresh wt.		
	construct	line no	GLUCOSE	FRUCTOSE	SUCROSE
Record	control	19	10.963135	7.9151729	7.3737653
Record	14272	24	3.013221	2.0279761	10.734599
Record	14272	34	4.5306819	3.2073172	10.639659
Record	14272	69	4.5224194	3.1380518	9.8068449

Year 2

Storage at 100°C for 12 weeks

			mean umol/gram fresh wt.		
	construct	line	GLUCOSE	FRUCTOSE	SUCROSE
Record	control	17	4.521635	2.09497	5.742809
Record	14272	24	0.636252	0.581957	7.727493
Record	14272	34	1.267106	0.726323	6.564545
Record	14272	69	0.573386	0.539863	6.72161

Storage at 60°C for 12 weeks

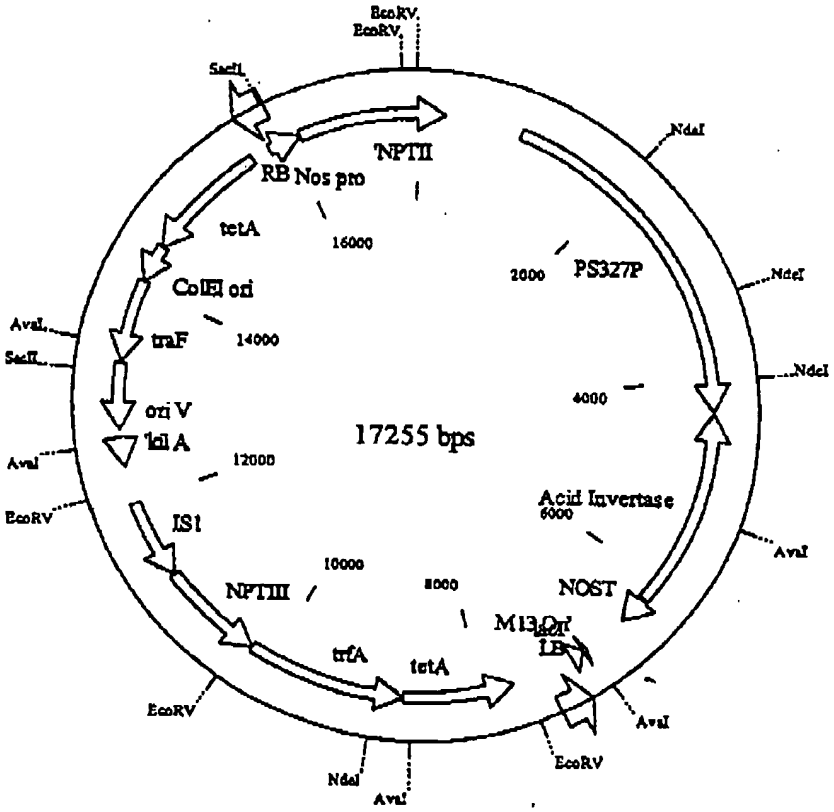
			mean umol/gram fresh wt.		
	construct	line	GLUCOSE	FRUCTOSE	SUCROSE
Record	control	17	9.864134	4.858925	7.090633
Record	14272	24	3.280316	1.65375	8.09423
Record	14272	34	3.017577	1.67629	7.238636
Record	14272	69	1.996818	1.210684	8.339795

TABLE 3. Modified Pyruvate Kinase Activity in Transgenic Plants

line	Activity nmol/min/gFWt	as % of control
control	938	100
antisense 3.29	156	16.7
antisense 5.40	334	35.6
antisense 5.42	508	54.1
sense 4.40	335	35.7
sense 4.53	393	41.9

FIGURE 1

pFW14272



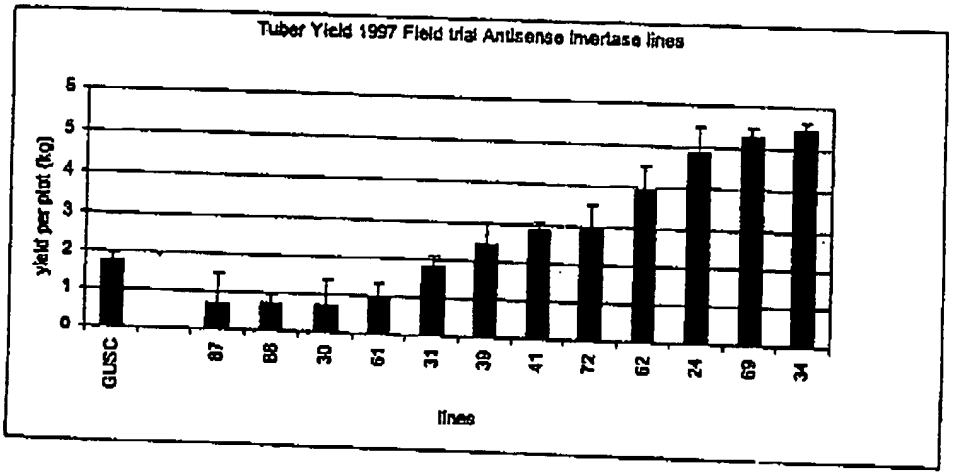

Tuber Yield 1997 Field trial Antisense invertase lines
yield per plot (kg)
0
1
2
3
4
5
6
GUSC
87
88
30
61
31
39
41
72
62
24
69
34
lines

FIGURE 2

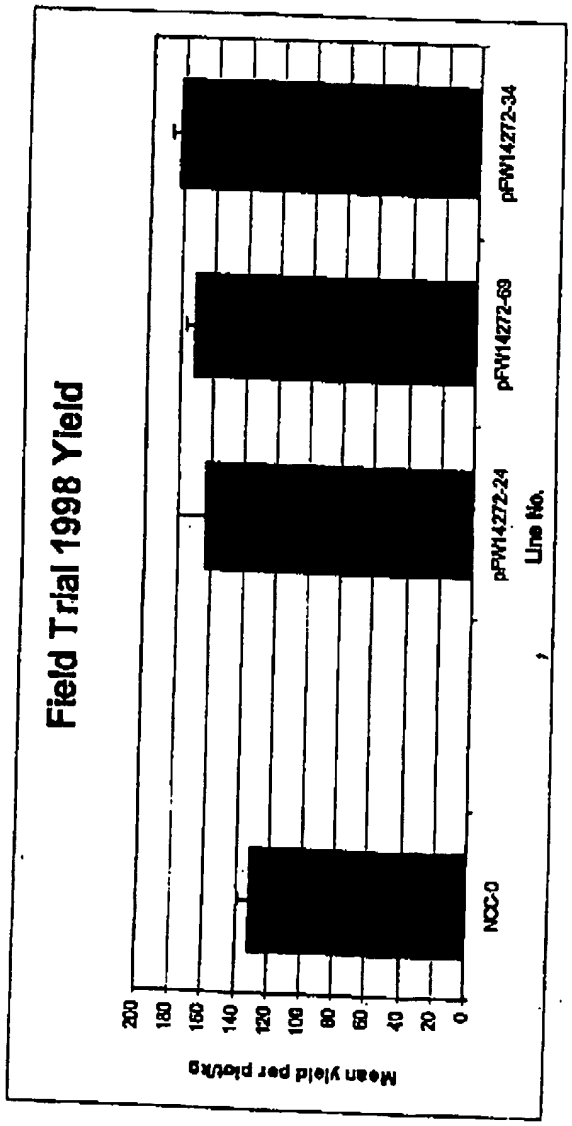
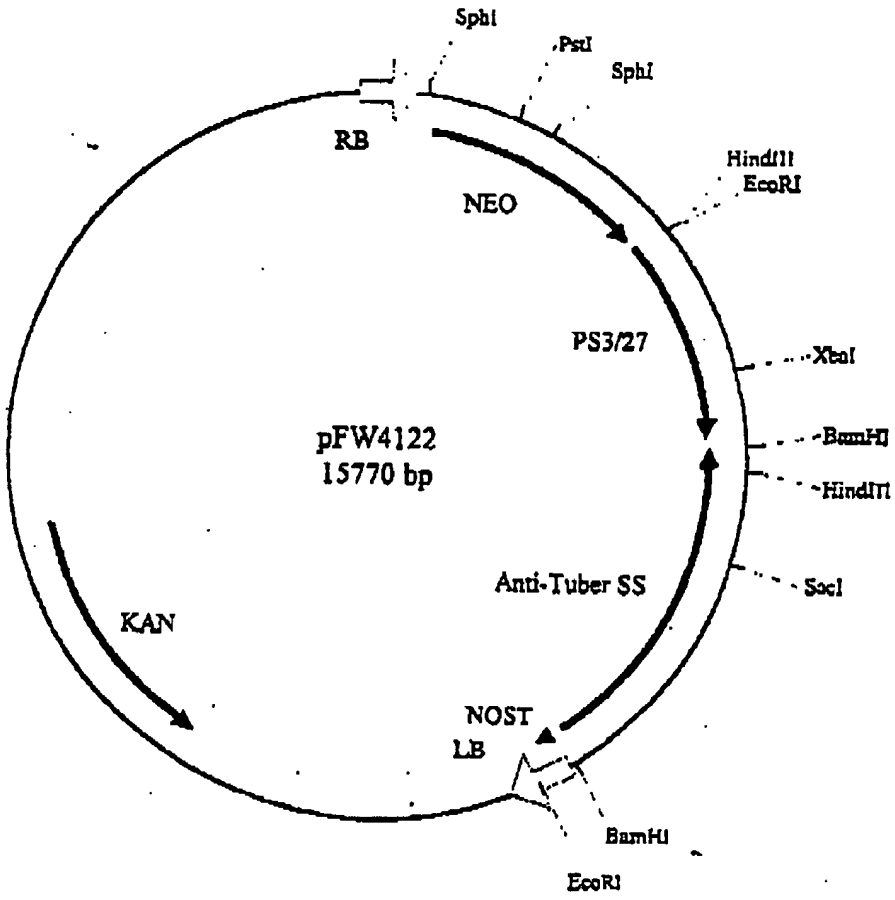

Field Trial 1998 Yield
Mean yield per plot/kg
200
180
160
140
120
100
80
60
40
20
0
NCC-0
pFW14272-24
pFW14272-69
pFW14272-34
Line No.

FIGURE 3

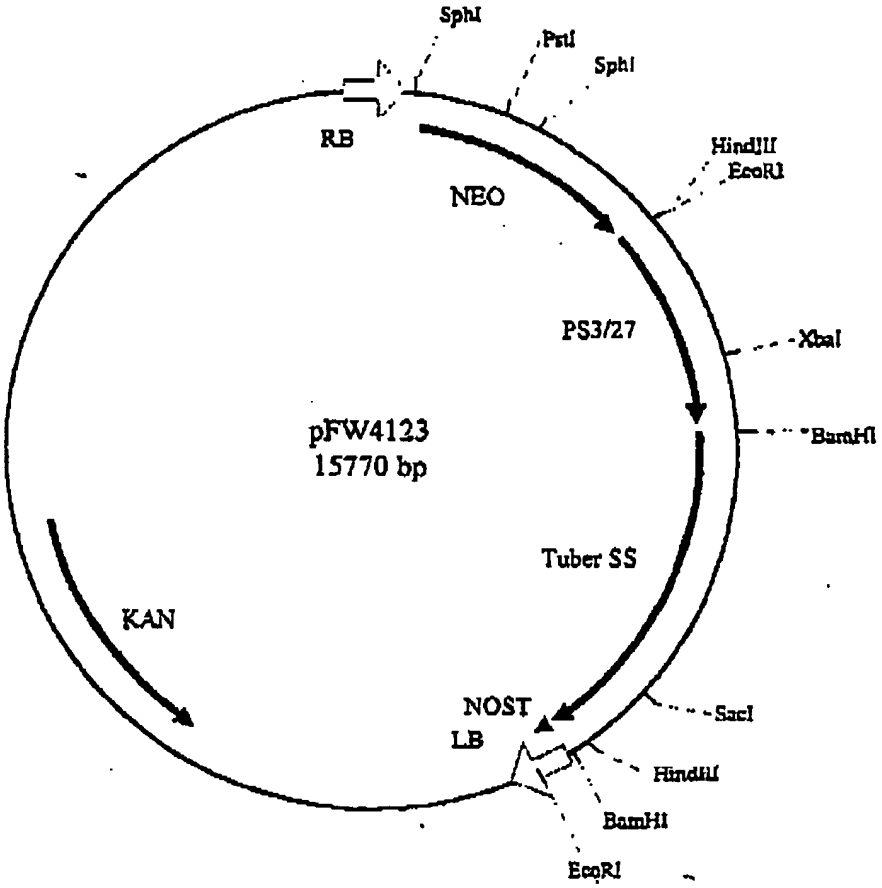
Patatin Antisense Tuber Sucrose Synthase Plasmid



A Bin19 based plasmid vector harbouring the tuber specific sucrose synthase isoform, *Sus4*, in antisense orientation, relative to the patatin promoter, *PS3.27*.

FIGURE 4

Patatin Sense Tuber Sucrose Synthase Plasmid



A Bin19 based plasmid vector harbouring the tuber specific sucrose synthase isoform, *Susd*, in the sense orientation, relative to the patatin promoter, *PS3/27*.

FIGURE 5

Modified Sucrose Synthase Activity in Transgenic Plants

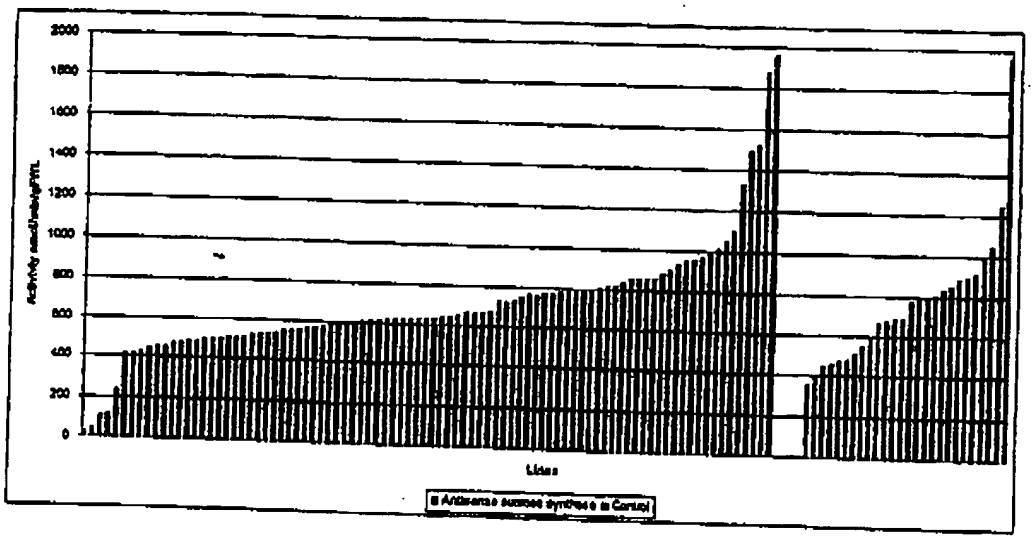
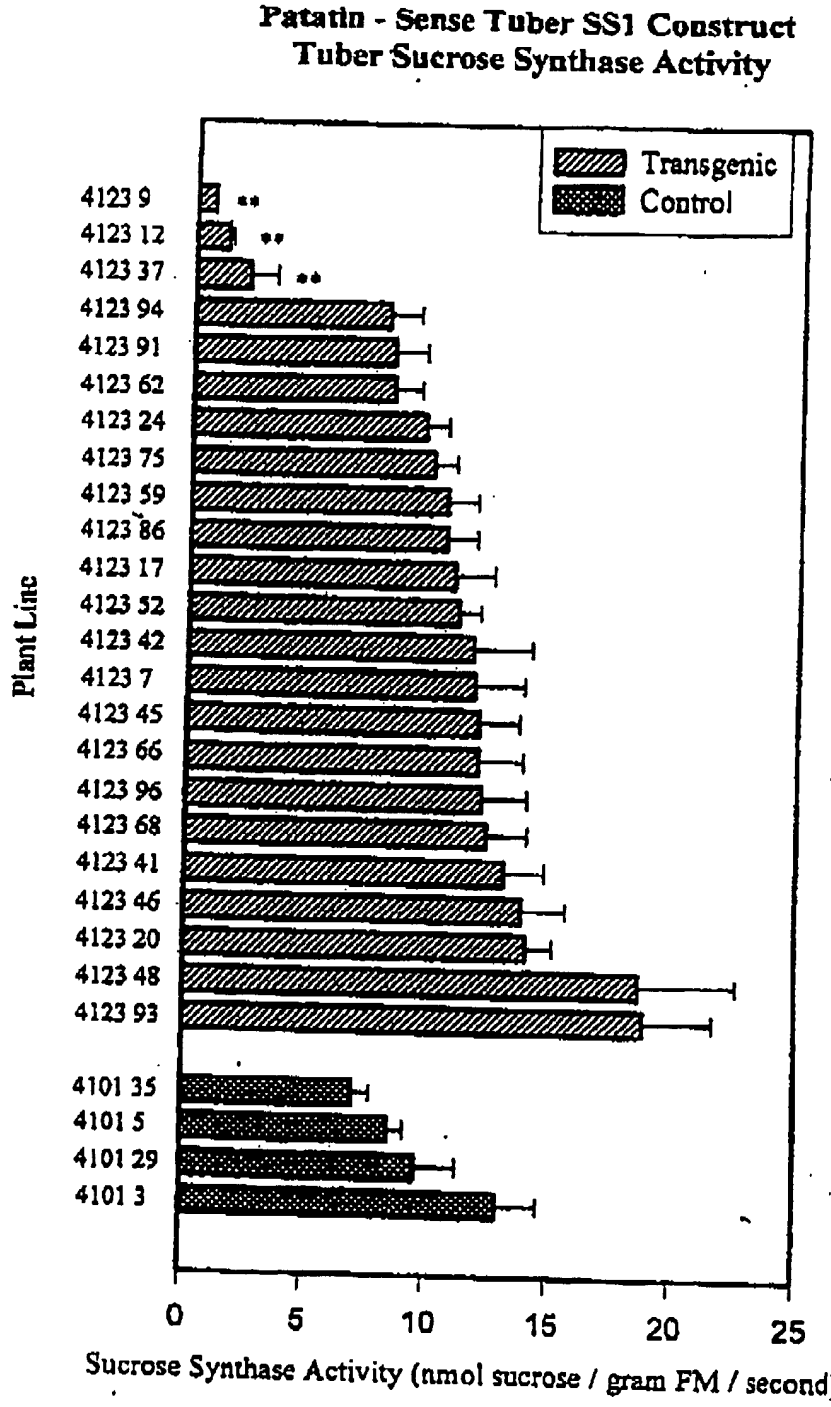


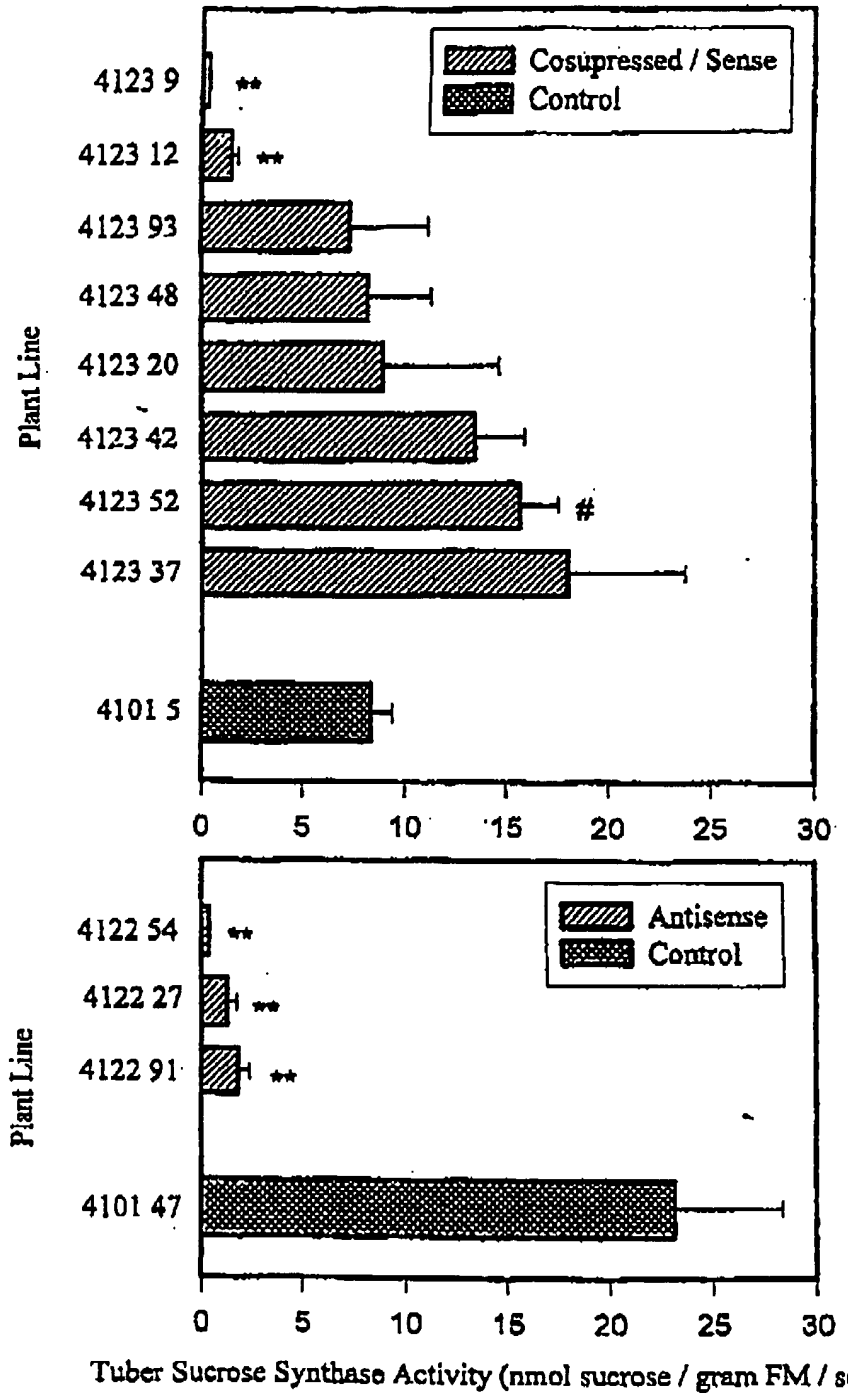
FIGURE 6



Each bar represents the mean and standard error of eighteen samples obtained from a single transformant. Tukey-HSD analysis indicates significantly reduced ** ($P < 0.01$) in tuber sucrose synthase activity relative to control lines.

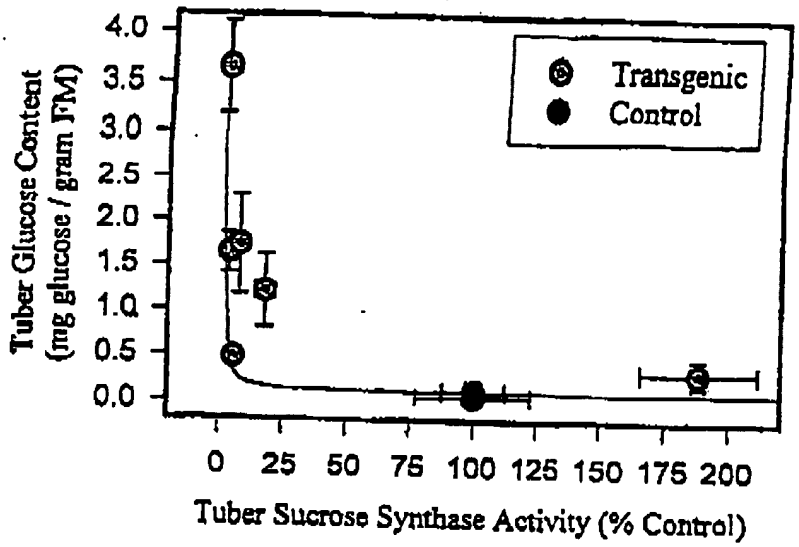
FIGURE 7

FIGURE 8 Tuber Sucrose Synthase Activity Screen



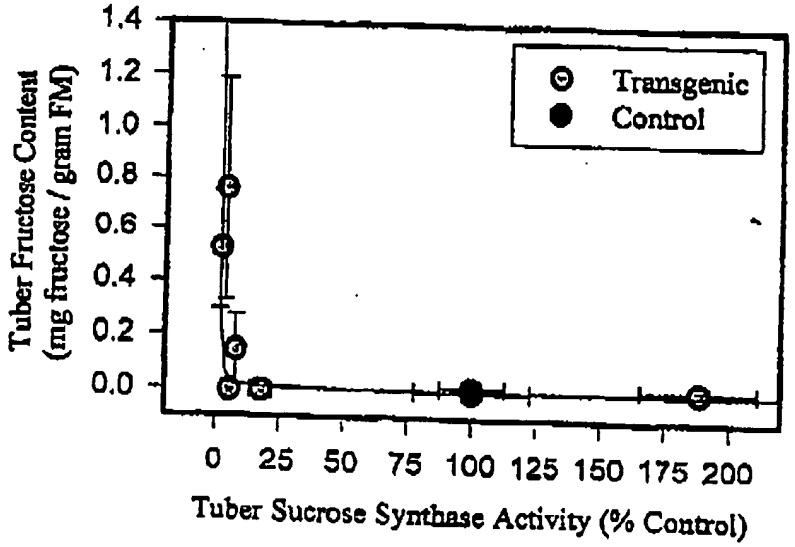
Each bar represents the mean and standard error of six replicate tuber samples from a single transgenic line. Separate variance t-test (Unistat) indicate significantly reduced ** ($P<0.01$) and significantly increased # ($P<0.05$) tuber sucrose synthase activity, relative to controls.

FIGURE 9A Tuber Glucose Content



Each point represents the mean and standard error of six replicate tuber samples obtained from a single transgenic or control line.

FIGURE 9B Tuber Fructose Content



Each point represents the mean and standard error of six replicate tuber samples obtained from a single transgenic or control line.

FIGURE 10A Potato Tuber Glucose-6-Phosphate

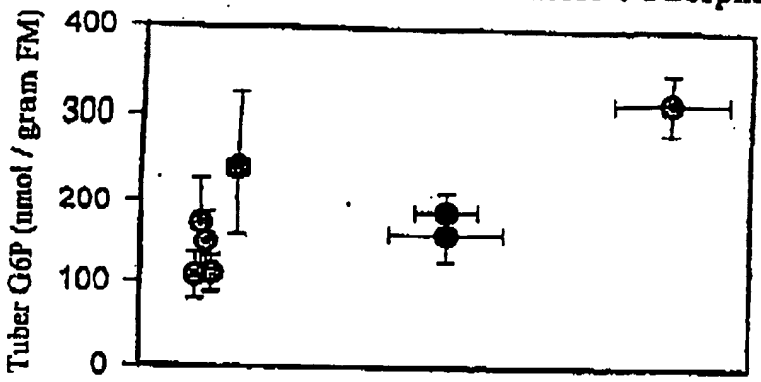


FIGURE 10B Potato Tuber Fructose-6-Phosphate

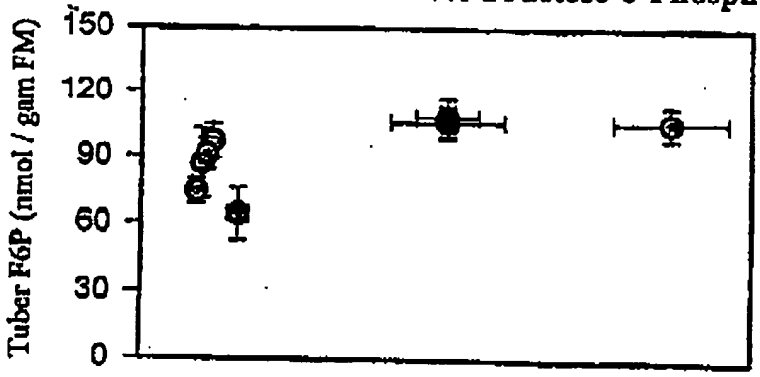
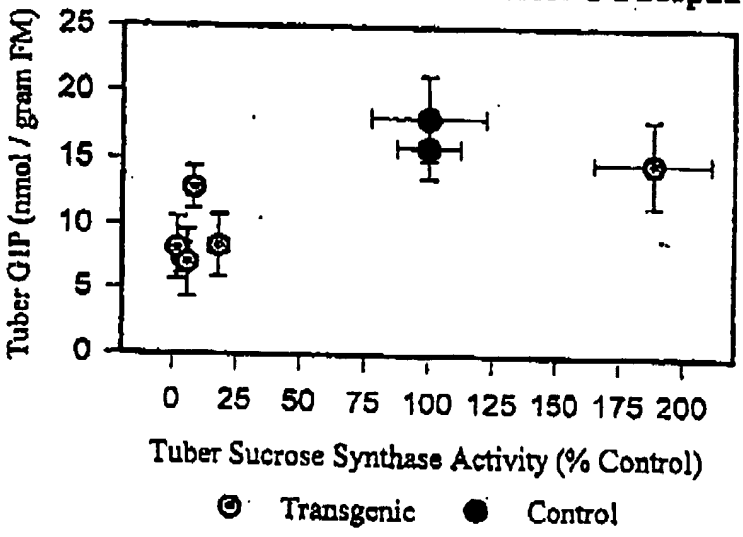
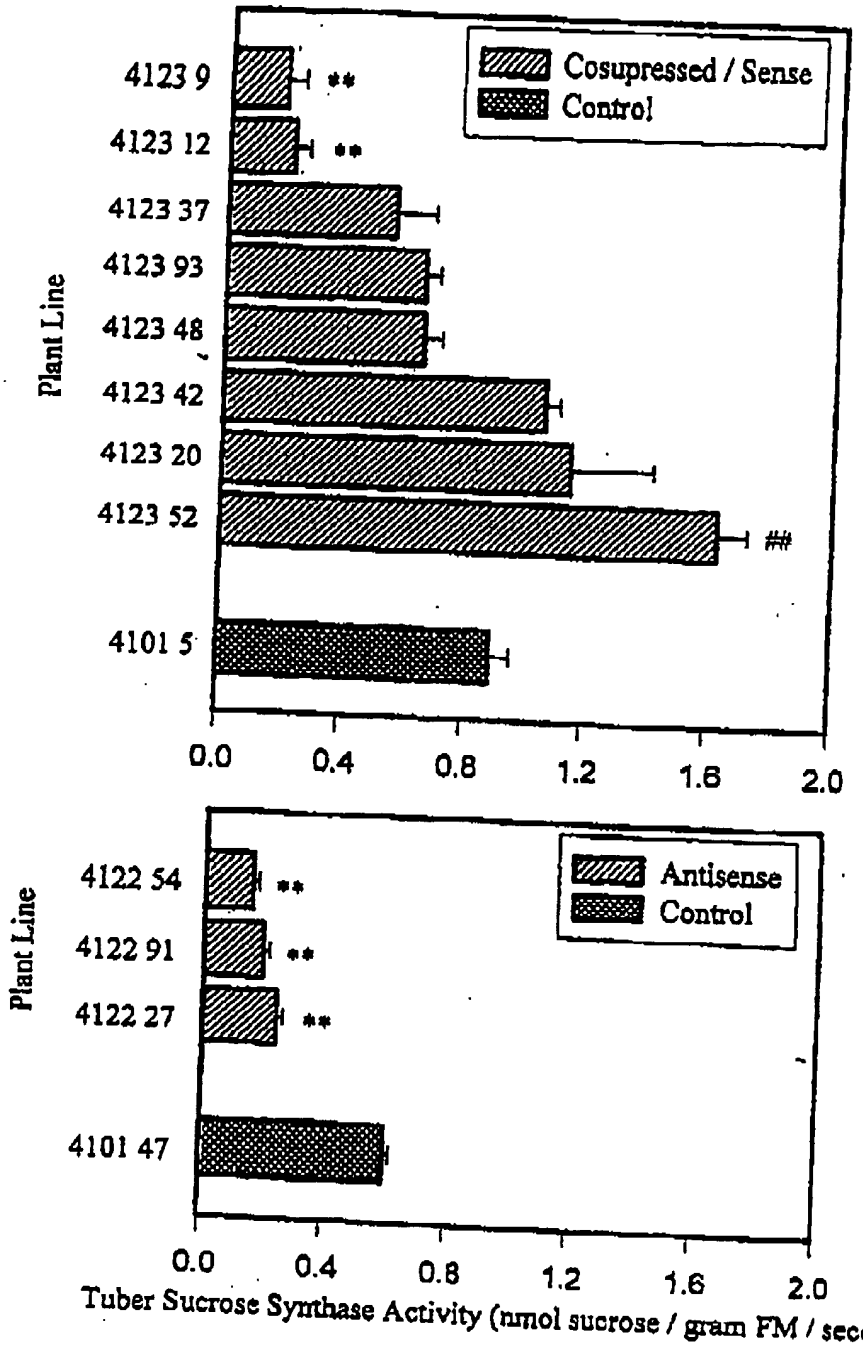


FIGURE 10C Potato Tuber Glucose-1-Phosphate



Each point represents the mean and standard error of six replicate tuber samples obtained from a single transgenic or control line.

FIGURE 11 Tuber Sucrose Synthase Activity Screen



Each bar represents the mean and standard error of six replicate tubers from each transgenicand control line. Tukey-HSD analysis indicates significantly reduced ** (P<0.01) and significantly increased ## (P<0.01) sucrose synthase activity relative to the control lines.

FIGURE 12A Tuber Glucose Content

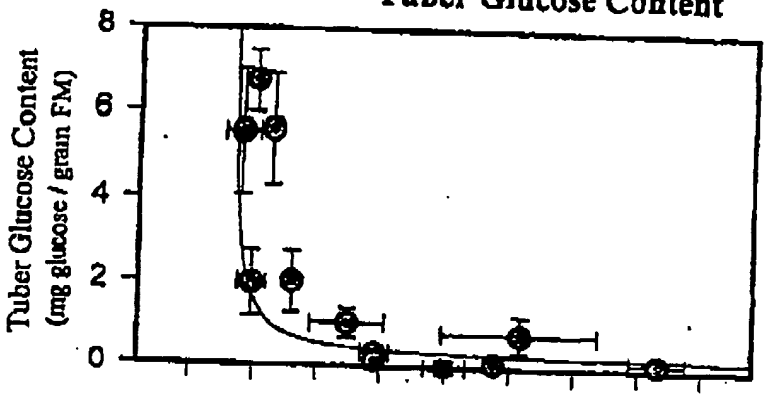


FIGURE 12B Tuber Fructose Content

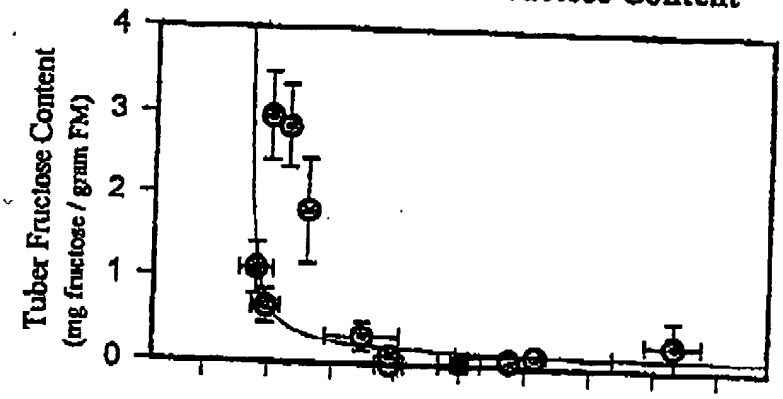
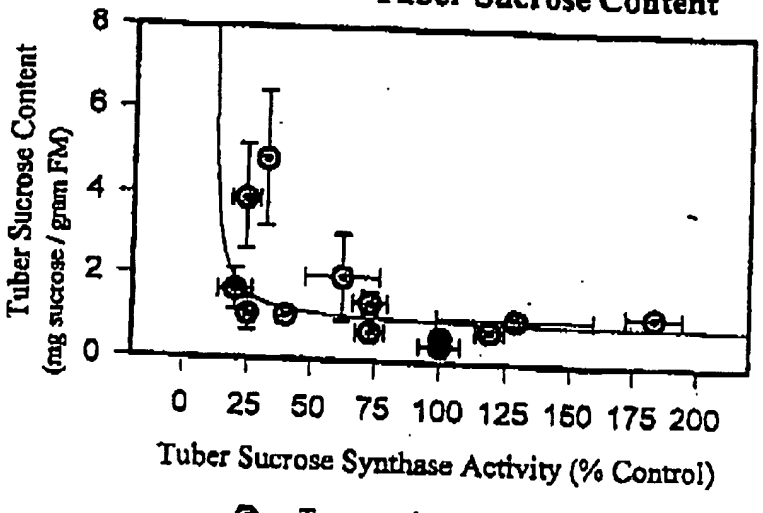


FIGURE 12C Tuber Sucrose Content



Each point represents the mean reducing sugar concentration and standard error for six replicate tuber samples from a single transgenic or control line.

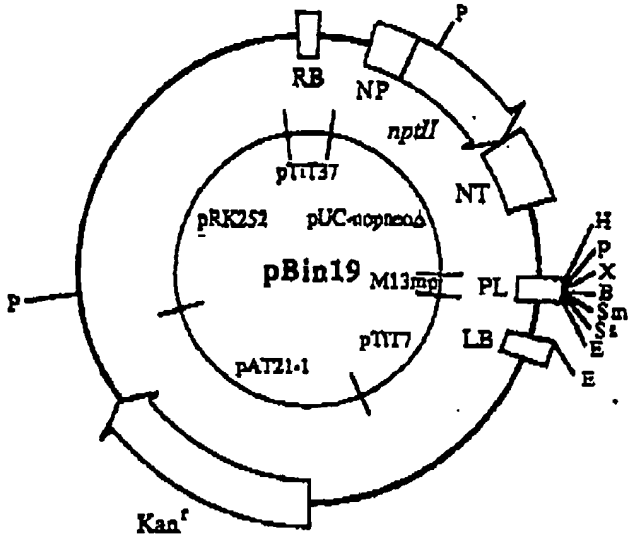


FIGURE 13A **The binary vector pBin19, its GUS-encoding derivative pFW4101, and three PK-34-containing vectors derived from pFW4101**

Maps are presented of the binary vector pBin19 (Bevan, 1984), and a GUS-encoding vector pFW4101 (Blundy *et al.*, 1991) which was derived from it. Furthermore, maps have been established of three plasmids derived from pFW4101 which contain the clone PK-34.

(a) pBin19 - a 10-kb binary vector designed for use in *Agrobacterium*-mediated plant transformation (Bevan, 1984). The vector was constructed from the plasmids indicated around the inner ring of the figure. These plasmids are described by Bevan (1984) and references therein;

(b) pFW4101 - contains the GUS coding sequence under the control of the patatin promoter PS3/27 (Wenzler *et al.*, 1989) and the nopaline synthase terminator region, inserted between the *Hind*III and *Bam*HI sites of the polylinker region of pBin19;

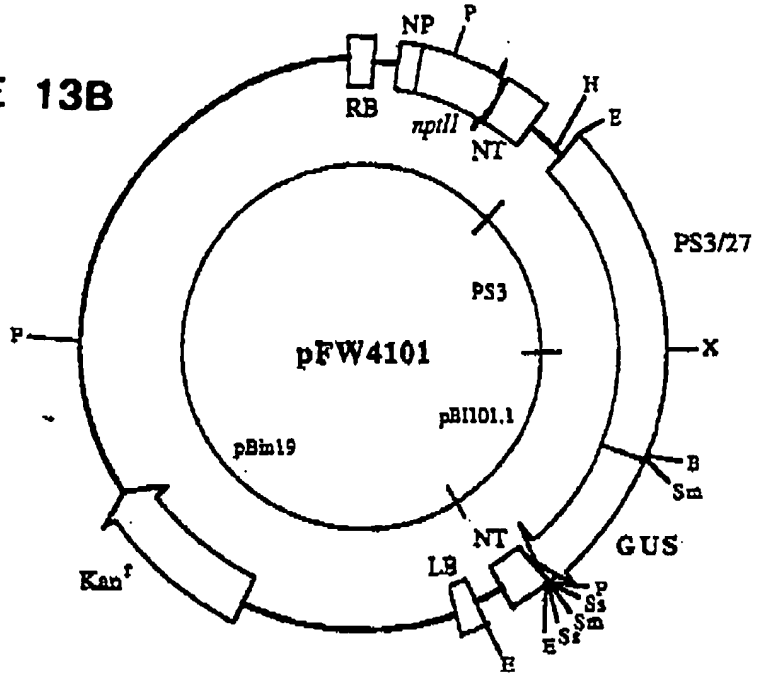
(c) pFW4111 - the GUS sequence was removed as a *Sma*I-*Sst*I fragment, and replaced by a truncated PK-34 clone in the antisense orientation (asPK-34');

(d) pFW4112 - full-length PK-34 clone in the antisense orientation in place of the GUS-sequence;

(e) pFW4113 - the GUS-sequence was replaced by a full-length PK-34 clone in the sense orientation.

Abbreviations: RB, right border of T-DNA; NP, nopaline synthase promoter; *nptII*, neomycin phosphotransferase type II from bacterial transposon Tn5; NT, nopaline synthase terminator region; PL, *lac* polylinker; LB, left border of T-DNA; GUS, β-glucoronidase; Kan^r, aminoglycoside phosphotransferase type III from *Streptococcus faecalis*; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Sm, *Sma*I; Sc, *Sst*I; X, *Xba*I.

FIGURE 13B



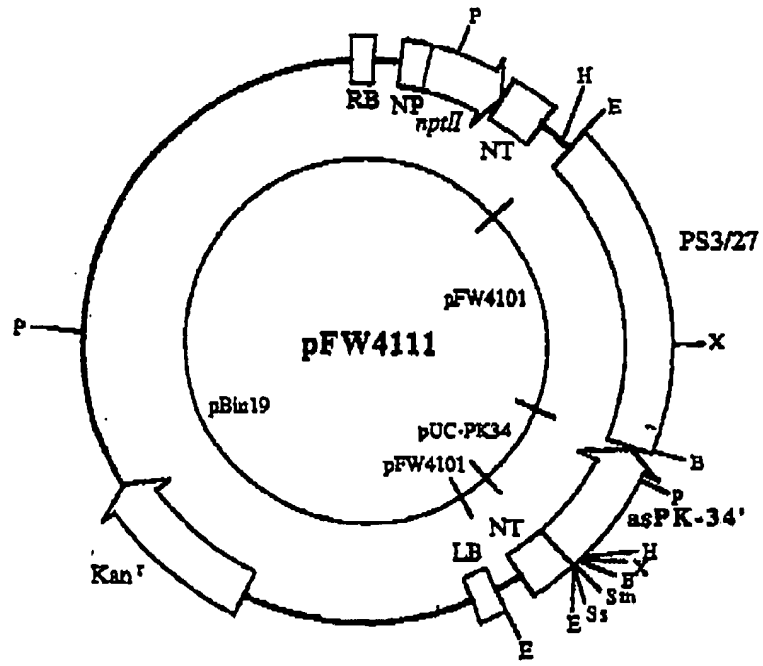
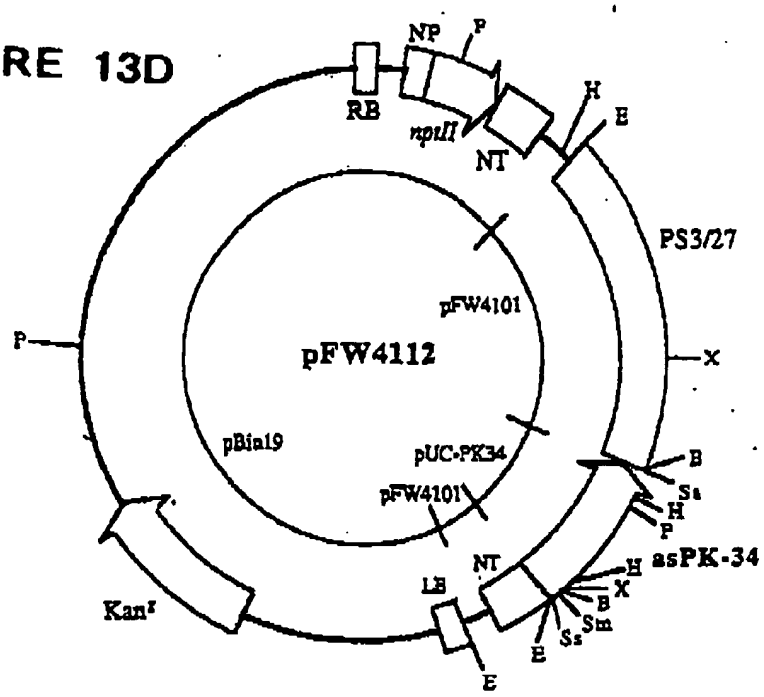


FIGURE 13C

FIGURE 13D



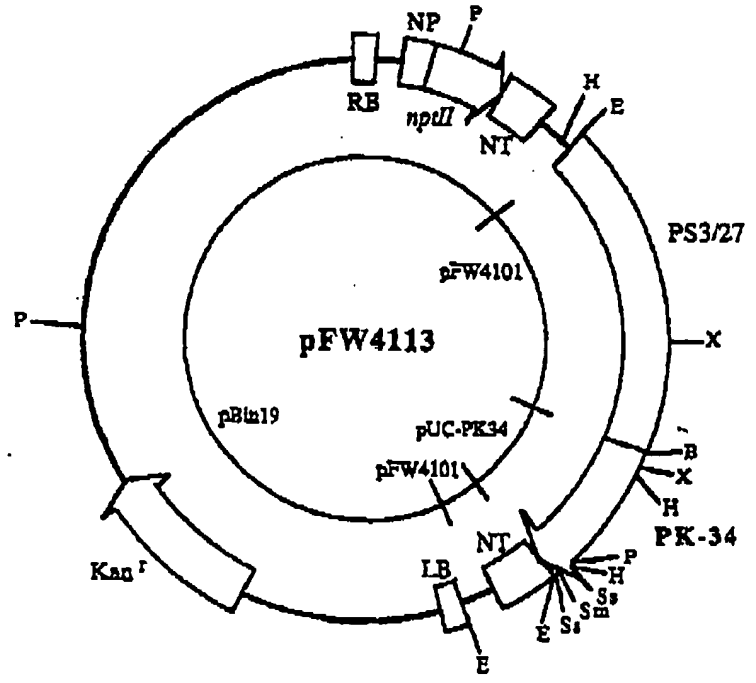


FIGURE 13E

FIGURE 14

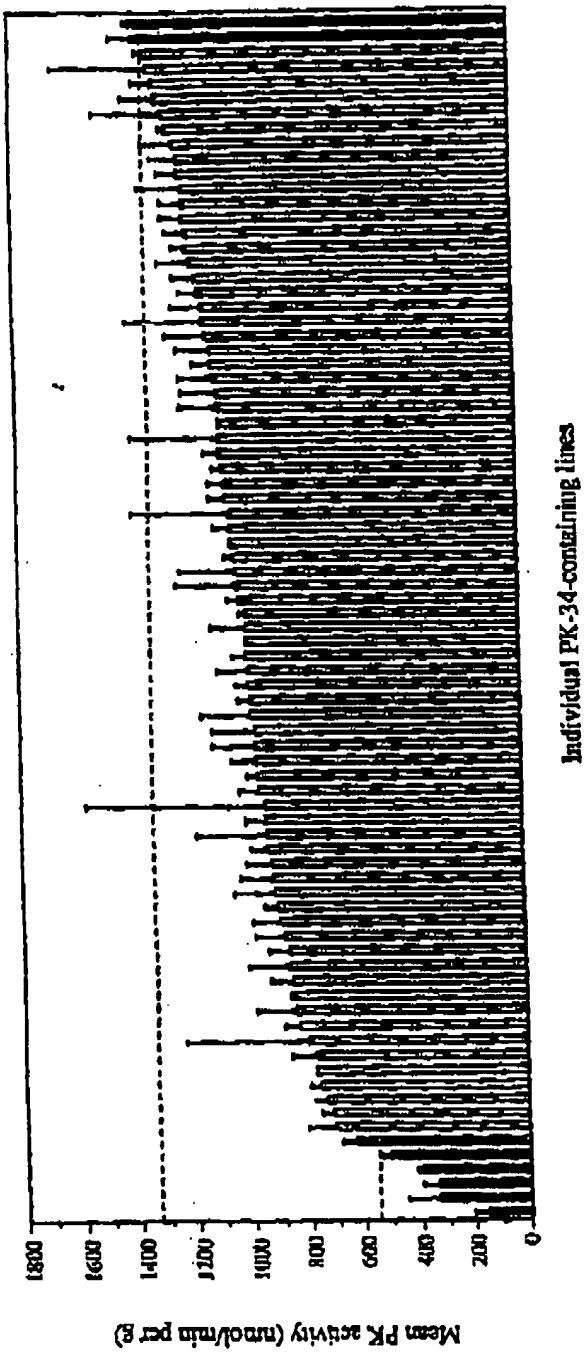


FIGURE 15A

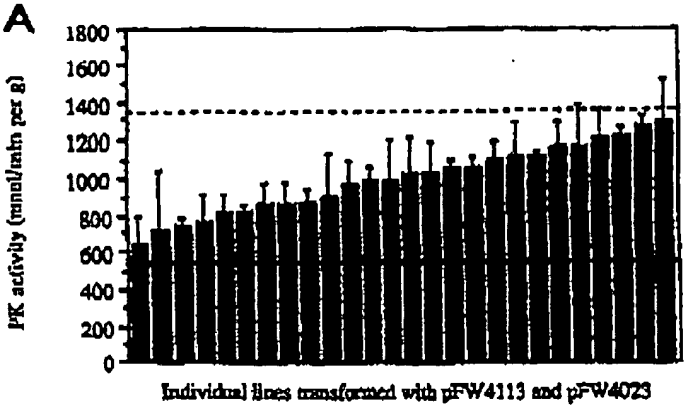
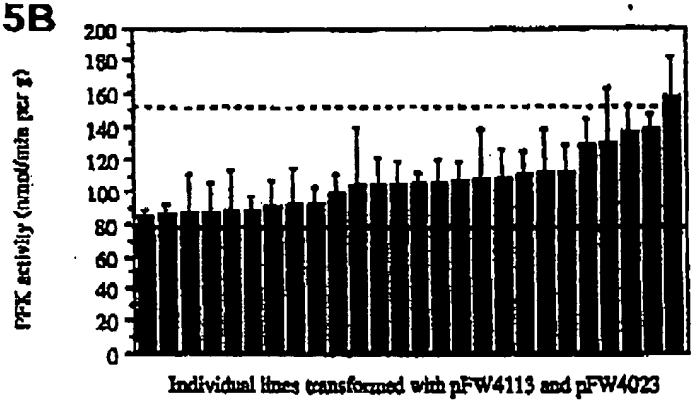


FIGURE 15B



Activity of PK and PFK(ATP) in microtubers from shoots transformed with the PK-encoding vector pFW4113 and the PFK(ATP)-encoding vector pFW4023

The maximum catalytic activities of PK (a) and PFK(ATP) (b) were measured in extracts of microtubers produced from shoots which were regenerated from leaf discs co-cultivated with the binary vectors pFW4113 (encoding potato cytosolic PK) and pFW4023 (encoding *E.coli* PFK(ATP)). Two, three, or four microtubers from each line were extracted as described in Section 2.8.b in 400 µl extraction buffer, and the extracts were kept on ice. Single samples were taken from each extract for the assay of each enzyme, and the mean activity of each enzyme for each line was calculated.
The figure shows the mean enzyme activities for 26 transgenic lines, arranged in order of increasing activity. Error bars represent the standard deviations of the means. A given line does not necessarily appear in the same position in (a) and (b). The horizontal lines represent 95% confidence limits of the means of the control enzyme activities.

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APPENDIX 3. MATERIALS AND METHODS

VECTORS AND TRANSFORMATION

Transformation with a PK construct was achieved using the binary vector pBIN19, its GUS-encoding derivative pFW4101, and three PK-34-containing vectors derived from pFW4101. The procedure used to produce a chimeric PK gene suitable for tuber-specific expression is shown in Figure 10a-e of the Declaration. Maps of the binary vector pBin19 (Bevan, 1984), and a GUS-encoding vector pFW4101 (Blundy *et al.*, 1991) which was derived from it, and maps of three plasmids derived from pFW4101 which contain the clone PK-34 are shown.

Figure 10a: pBin19 - a 10-kb binary vector designed for use in *Agrobacterium*-mediated plant transformation (Bevan, 1984). The vector was constructed from the plasmids indicated around the inner ring of the figure. These plasmids are described by Bevan (1984) and references therein;

Figure 10b: pFW4101 - contains the GUS encoding sequence under the control of the patatin promoter PS3/27 (Wenzler *et al.*, 1989) and the nopaline synthase terminator region, inserted between the *Hind*III and *Bam*HI sites of the polylinker region of pBin 19;

Figure 10c: pFW4111- the GUS sequence was removed as a *Sma*I-*Sst*I fragment, and replaced by a truncated PK-34 clone in the antisense orientation (asPK-34');

Figure 10d: pFW4112 - full-length PK-34 clone in the antisense orientation in place of the GUS-sequence;

Figure 10e: pFW4113 - the GUS-sequence was replaced by a full-length PK-34 clone in the sense orientation. Abbreviations: RB, right border of T-DNA; NP, nopaline synthase promoter; *nptII*, neomycin phosphotransferase type II from bacterial transposon Tn5; NT, nopaline synthase terminator region; PL, *lac* polylinker; LB, left border of T-DNA; GUS, β-glucoronidase; Kan^r, aminoglycoside phosphotransferase type III from *Streptococcus faecalis*; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; Sm. *Sma*I; Ss, *Sst*I; X, *Xba*I.

The method described above for making vectors comprising PK encoding sequences are also applicable to sequence encoding acid invertase (pFW14272, Figure 1 of Declaration), starch synthase, 6-phosphofructokinase, sucrose synthase, and/or sucrose phosphate synthetase.

The vectors comprising sequences encoding the above-mentioned enzymes were readily transferred into *Agrobacterium tumefaciens* by triparental mating. *Agrobacterium* comprising the constructs were used to transform plant tissues, leaf discs, calli, and microtubers, *in vitro*.

Transformation of potato

Solanum tuberosum was transformed with pFW14272 using the method of leaf disk cocultivation essentially as described by Horsch et al. (Science 227: 1229-1231, 1985). The youngest two fully-expanded leaves from a 5-6 week old soil grown potato plant were excised and surface sterilised by immersing the leaves in 8% 'Domestos' for 10 minutes. The leaves were then rinsed four times in sterile distilled water. Discs were cut from along the lateral vein of the leaves using a No. 6 cork borer. The discs were placed in a suspension of

Agrobacterium, containing the plasmid listed above for approximately 2 minutes. The leaf discs are removed from the suspension, blotted dry and placed on petri dishes (10 leaf discs/plate) containing callusing medium (Murashige and Skoog (MS) agar containing 2.5μg/ml BAP, 1 μg/ml dimethylaminopurine, 3% (w/v) glucose).

After 2 days the discs were transferred onto callusing medium containing 500μg/ml Claforan and 50μg/ml Kanamycin. After a further 7 days the discs were transferred (5 leaf discs/plate) to shoot regeneration medium consisting of MS agar containing 2.5μg/ml BAP, 10 μg/ml GA3, 500μg/ml Claforan ,50μg/ml Kanamycin and 3% (w/v) glucose. The discs were transferred to fresh shoot regeneration media every 14 days until shoots appeared. The callus and shoots were excised and placed in liquid MS medium containing 500μg/ml Claforan and 3% (w/v) glucose.

Rooted plants were weaned into soil and grown up under greenhouse conditions to provide tuber material for analysis. Alternatively microtubers were produced by taking nodal pieces of tissue culture grown plants onto MS agar containing 2.5μg/ml Kanamycin and 6% (w/v) sucrose. These were placed in the dark at 19°C for 4-6 weeks when microtubers were produced in the leaf axils.

PROPAGATION CONDITIONS FOR TRANSGENIC PLANTS

To examine the influence of sucrose synthase activity and other enzymes on the amount of metabolic intermediates in the starch and sugar biosynthesis/degradation pathways, transformed and non-transformed control potato plants were propagated in both greenhouse and field conditions. Greenhouse conditions consisted of propagating potato

plants in 2 litre pots containing Levington's M3 compost, under controlled greenhouse conditions: 16°C to 25°C and ambient light supplemented by a 12 hour photoperiod of quantum irradiance 300 umol/m^2/second. Standard agricultural practices were employed to propagate potato plants in the field.

EXTRACTION OF POTATO TUBERS AND LEAVES FOR ENZYME ASSAYS

Extraction of tissue from potato tubers, microtubers, and leaves was carried out in essentially the same way in order to assay enzyme maximum catalytic activities. All buffers used were ice-cold, and all procedures were carried out at 4°C unless otherwise stated. The extraction buffer contained 50 mM-Hepes (pH 7.5); 30 mM-KCl; 6 mM-$MgCl_2$; 1 mM-EDTA; 10 mM-DTT; 5% (w/v) insoluble PVPP. Tubers (>0.5 g) and leaf material were ground using a pestle and mortar in about 5 volumes extraction buffer. The sample was then transferred to a ground-glass homogeniser and ground thoroughly. The cellular debris and insoluble material was collected without delay by centrifugation at 12000g for 5 min, and enzyme activity was assayed in the supernatant.

The extraction of microtubers and small (<0.5 g) greenhouse-grown tubers was carried out at room temperature in 1.5-ml Eppendorf tubes. The tissue was added to 0.4 ml ice-cold extraction buffer, and macerated using a disposable grinder for 30 s. The cellular debris and insoluble material was removed by centrifugation at 12000g for 3 min, and the supernatant was decanted into a fresh tube and kept on ice. Enzyme activities were assayed in the supernatant.

DETERMINATION OF ENZYME ACTIVITIES

ASSAY OF PYRUVATE KINASE

Pyruvate kinase activity was measured at 25°C by coupling the reaction to lactate dehydrogenase. The method used was a modification of that of Bucher and Pfleiderer (1955). The reaction mixture contained in 1 ml: 50 □mol Mops-NaOH (pH 7.0), 100 □mol KCl, 15 □mol $MgCl_2$, 0.15 □mol NADH, 0.15 units hog muscle lactate dehydrogenase, and 1 □mol ADP (di-monocyclohexylammonium salt). The endogenous rate of NADH oxidation was measured for 4 min before starting the reaction. The reaction was started by adding 5 □mol PEP (trisodium salt). A control reaction containing no ADP was used to determine the rate of PEP consumption in the absence of this substrate. Subtraction of the reaction rate in the absence of ADP gave the actual pyruvate kinase activity, corrected for the presence of PEP phosphatase activity. The reaction rate was proportional to the amount of enzyme over the range employed, and was linear with time for at least 20 min. One unit of enzyme activity is defined as the amount which catalyses the formation of 1 □mol pyruvate min^{-1}. Specific activity is given as units mg^{-1} protein.

INVERTASE STOPPED LINKED ASSAY

Extraction

Ten grams of tuber were mashed in a chilled pestle and mortar with 2.5g of PVPP and 50 ml of extraction buffer was added. The mixture was transferred to a blender and blended for 30 sec. The mixture was then transferred to an Oakridge centrifuge tube and spun at 10 K for 20 min 4°C. Aliquots of the s/n were spun in a microfuge at 4°C for 5 min. Then kept on ice.

De-salting extracts:

A PD10 column (Pharmacia) was equilibrated with 25 ml of extraction buffer. 2.5 ml of extract was added, the mixture was eluted with 3.5 ml of extraction buffer.

Stage 1

One hundred microlitres of de-salted extract with 100μl of stage 1 buffer (Eppendorph tubes on ice) was used. Four replicates and a extraction buffer plus stage 1 buffer blank were used per time point. Time points used were 10, 20, 30, 40, 50, and 60 min. (A positive control with commercial invertase was also used with a 20 min incubation. Stock Invertase was 3 units/μl dilute 10 μl in 90 μl of extraction buffer and diluted 16.7 μl of this in 50ml ext. buffer use 100 μl with stage 1 buffer). Incubation of assays was at 37 °C, time points were stopped by boiling for 2 min then placed on ice. Centrifuge time points were 2 min in a microfuge at full speed to remove precipitated protein. Samples were keep on ice for stage 2.

Stage 2: The following were combined in a plastic cuvette sequentially.

850 μl 2x stage II buffer

100 μl from stage I time point

10 μl 10mM NAD

10 μl Hexokinase (20 μl of 1.5 u/μl stock dil in 320 μl of 2x stage II buffer)

10 ul PGI (20 μl of 3.4 u/μl stock dil in 320 μl of 2x stage)

10 ul G6PDH (20 μl of 10u/μl stock dil in 320 μl of 2x stage)

Spectrophotometer readings were recorded beginning at 340nm (no ref) to establish a baseline (@ 1.5 min). The assay was started by the addition of 10 μl of 100mM ATP.

After 9 min the increase in absorbance stopped. The table of values from which the delta OD was calculated was printed.

Buffers

Extraction buffer:

200mM NaOAc pH 4.7

2 mM $MgCl_2$

10 mM DTT

Stage I buffer:

100 mM NaOAc pH 4.7

100 mM Sucrose

2X Stage II buffer:

100mM Tris pH 8.1

5 mM $MgCl_2$

SUCROSE SYNTHASE ASSAY

One hundred miligrams of potato tuber tissue (powdered in liquid nitrogen) was extracted in 1 ml of ice cold extraction buffer (50 mM Hepes-KOH pH 7.4; 5 mM $MgCl_2$; 1 mM EDTA; 1mM EGTA; 10% (v/v) glycerol; 5 mM $NaHCO_3$; 10 mM DTT; 2 mM benzamidine; 5nM PMSF; 2 mM α-caprionic acid; 1% (w/v) PVP). Samples were clarified

by centrifuging at 14000rpm for 5 minutes at 4°C. The resulting supernatant was desalted using PD10 columns (from Pharmacia).

Sucrose synthase activity was assayed by taking aliquots (20 μl) of the desalted extract in a final volume of 100μl containing 20 mM Hepes-KOH pH 7.0, 100mM sucrose and 4 mM UDP which was then incubated at 25°C for 30 minutes. Reactions were terminated by heating at 95°C for 3 minutes. The production of UDPglucose in these reactions was determined spectrophotometrically at 340 nm in a final volume of 1 ml which contained 200 mM glycine-KOH pH 8.9; 5 mM $MgCl_2$; 1mM NAD and 0.01 units of UDPglucose dehydrogenase.

ASSAY OF OTHER ENZYMES

Other enzymes were measured in essentially the same way, *mutatis mutandis*, as pyruvate kinase. Coupling enzymes were from rabbit muscle unless otherwise indicated. All assay mixtures were optimised for potato tuber by Morrell (1984) unless otherwise stated, and were carried out at 25°C. For example, PFK (E.C. 2.7.1.11) activity was assayed according to the teachings of Wong and ap Rees (1971) and the following conditions: 50 mol Tris-HCl (pH 7.4); 0.5 □mol ATP (disodium salt); 0.15 □mol NADH; 1 □mol MgCl2; 0.135 units aldolase; 0.12 units triose-phosphate isomerase; 0.36 units -glycerol-phosphate dehydrogenase; 20 □mol fructose 6-phosphate.

LARGE SCALE EXTRACTION OF SUGARS FROM FRESH POTATO TUBERS

This extraction method was used for screening exercises where large numbers of samples were involved. Extracts were analysed for Glucose, Fructose and Sucrose using the enzyme linked spectrophotometric assay method. Extracts produced by this method are not suitable for the HPLC method of sugars analysis.

Materials Required

Sodium hydroxide

Glacial acetic acid

Elga water

Solution

33.5g Sodium hydroxide

Dissolve in 25L Elga water

Adjust the pH to 4.6 with Glacial acetic acid. (Approximately 96ml is needed).

Method

The potato tubers were washed thoroughly in tap water. The tubers were sliced into small pieces capable of homogenisation. One kilogram of chopped tubers were placed into a 2L plastic jug. The actual weight was recorded. Extraction solution was added to the chopped tubers until the total volume was 2L. The sample was transferred to a Waring

blender and the tuber and buffer were homogenised for 1 minute at high speed. The mixture was transferred to a plastic beaker and allowed to settle for approximately 5 minutes. Twenty-five millilitres of the suspension was pipetted from the blender into a boiling tube. The boiling tube was heated in boiling water for 20 minutes. The contents of the boiling tube were filtered through a Whatman's No. 1 filter paper into a clean sample tube. The extract was cooled to room temperature and analysed immediately, thought if this is not practicable, extracts can be frozen and stored so long as thawed extracts are thoroughly mixed before analysis.

DETERMINATION OF GLUCOSE, FRUCTOSE AND SUCROSE BY ENZYME LINKED SPECTROPHOTOMETRIC ASSAY

Used in conjunction with the large scale extraction method for sugar determinations in potato tubers.

Materials required

HEPES

Magnesium Chloride ($MgCl_2$)

β-Nicotinamide Adenine Dinucleotide (NAD)

Adenosine Triphosphate (ATP)

Elga Water

Enzymes required

Hexokinase

Glucose-6-Phosphate Dehydrogenase (G6PDH)

Phosphoglucose Isomerase (PGI)

Invertase

Preparation of Assay Cocktail:

A stock solution was prepared: 100mM in HEPES, pH 8.0 and 5mM in $MgCl_2$. Prior to analysis, NAD was added to a concentration of 4mM and ATP to a concentration of 1mM to the required quantity of the stock HEPES/$MgCl_2$ buffer. (Allow about 1.2 ml per assay).

Preparation of Enzyme Solutions:

One hundred and forty microlitres of stock hexokinase enzyme was used and the volume was made up to 1.0ml with Elga water. Stock G6PDH enzyme, 60μl, was used and the volume was made up to 1.0ml with Elga water. Stock PGI enzyme, 180μl, was used and the volume was made up to 1.0ml with Elga water. Invertase was used neat from the stock bottle.

Method

The filtered extracts were thawed and inverted several times to mix. Twenty microliters of extract supernatant were placed in a cuvette and 980μl of assay cocktail was added and mixed. The absorbance was measured at 340nm and the result was recorded when the reading had stabilised. Ten microliters of hexokinase solution was added, mixed, and the absorbance was recorded at 340nm when the reading had stabilised. Ten microliters of

G6PDH solution was added, mixed and the absorbance was recorded at 340nm when the reading has stabilised. Ten microliters of PGI solution was added, mixed and the absorbance was recorded at 340nm when the reading has stabilised. Thirty microliters of Invertase was added, mixed and the absorbance was recorded at 340nm when the reading has stabilised.

Calculation of Results:

GLUCOSE: μmol per assay = Δ O.D. due to the addition of G6PDH/6.22

FRUCTOSE: μmol per assay = Δ O.D. due to the addition of PGI/6.22

SUCROSE: μmol per assay = Δ O.D due to the addition of Invertase/(6.22*0.5)